PATENT ABSTRACTS OF JAPAN

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(54) **FREEZING DAMAGE-PROTECTING AGENT AND FREEZING AND STORING METHOD**

(57)Abstract:
PURPOSE: To obtain a freezing damage-protecting agent capable of suppressing hindrance due to freeze and improving survival rate in freezing a cell of a microorganism, a plant or an animal and having its high serviceability in fields of food industry, medicine industry, etc.
CONSTITUTION: This freezing damage-protecting agent for microorganisms or cells contains inulin type fructan as an active ingredient. The method for carrying out freeze storage of a microorganism or a cell is to immerse the microorganism or the cell in a solution containing the inulin type fructan and then freeze or lyophilize the microorganism or the cell.

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CLAIMS

[Claim(s)]
[Claim 1] The frost damage protective agent for the microorganism characterized by containing an inulin mold fructan as an active principle, or a cell.
[Claim 2] The frost damage protective agent according to claim 1 whose inulin mold fructan is the mixture of polymerization degree 3-6.
[Claim 3] The frost damage protective agent according to claim 1 whose inulin mold fructan is either of the polymerization degree 3, 4, and 5.
[Claim 4] The frost damage protective agent according to claim 1 a microorganism or whose cell is a cultured cell of lactobacillus bifidus, lactic acid bacteria, Escherichia coli, a Bacillus subtilis, yeast or vegetation, and an animal.
[Claim 5] The microorganism characterized by making it freeze or freeze-dry after dipping a microorganism or a cell into the solution containing an inulin mold fructan, or the cryopreservation approach of a cell.
[Claim 6] The cryopreservation approach according to claim 5 that inulin mold fructans are polymerization degree 3-6.
[Claim 7] The cryopreservation approach according to claim 5 that an inulin mold fructan is either of the polymerization degree 3, 4, and 5.
[Claim 8] The cryopreservation approach according to claim 5 that a microorganism or a cell is a cultured cell of lactobacillus bifidus, lactic acid bacteria, Escherichia coli, a Bacillus subtilis, yeast or vegetation, and an animal.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] This invention relates to the frost damage protective agent and the cryopreservation approach of making an inulin mold fructan an active principle. Furthermore, the freezing failure encountered in it when freezing the cultured cell of microorganisms, such as lactobacillus bifidus, lactic acid bacteria, and Escherichia coli, or vegetation, and an animal in a detail is controlled, and it is related with the frost damage protective agent and the cryopreservation approach of raising a survival rate.
[0002]
[Description of the Prior Art] Storage and preservation of a microorganism or a cell are not only used for preservation of the network which was only excellent, but the use range is expanding it. For example, by the microorganism, pressure of business of lactobacillus bifidus, the lactic acid bacteria, etc. is carried out as a food material in which the lyophilized cell has the ready intestines effectiveness. Moreover, preservation of the Escherichia coli introduced in the specific gene has been an important technical problem on gene engineering.

[0003] Generally, cryopreservation is performed for long term storage and preservation of living body liquid, such as a microorganism and a cell. After diluting with the frost damage protective agent which can be received physiologically in such cryopreservation conventionally, freeze storage and the approach of saving are almost the case. However, freezing is cruel for a living thing and the survival rate is reduced in many cases for the thermal impact produced at freezing and a fusion process, or crystal generation. Therefore, it was anxious for development of the frost damage protective agent which raises the survival rate after cryopreservation more, and the cryopreservation approach.
[0004] In order to control the frost damage of a microorganism or a cell, the approach of diluting with the protective agent which can be received physiologically was examined, and it was found out that glycerol is effective (Polge, Nature (Nature), 164 volumes, 666 pages, 1949].). Development of the protective agent for controlling frost damage also after that is performed. For example, in lyophilized-products-izing of lactobacillus bifidus or lactic acid bacteria, the approach using lactulose (JP,52-151787,A), vitamin E (JP,53-5747,B), corn steep liquor (JP,58-104787,A), raw starch (JP,58-149675,A), and a cyclodextrin (JP,63-251080,A) as a protective agent added in order to raise a survival rate is learned. However, using this protective agent in large quantities, the matter of these approaches was expensive, and they had the trouble that palatability was not desirable etc. Moreover, when carrying out cryopreservation of the animal cell, the approach of needing addition of a blood serum in the freezing culture medium, or adding methyl cellulose, trehalose (Patent Publication Heisei No. 501112 [four to] official report) or trehalose, and gelatin (JP,5-7489,A) as a protective agent instead of a blood serum is also established. However, also in these approaches, improvement in a survival rate has been a technical problem.
[0005]
[Means for Solving the Problem] Then, this invention persons completed a header and this invention for the ability of extinction of a microorganism or a cell to be remarkably controlled at the time of cryopreservation or freeze drying by using an inulin mold fructan as a frost damage protective agent in a diluent, as a result of examining many things, in order to control extinction of the microorganism in a freezing process, or a cell.
[0006] After dipping a microorganism or a cell into the solution which contains an inulin mold fructan in the frost damage protective agent list for the microorganism characterized by this invention containing an inulin mold fructan as an active principle, or a cell, the cryopreservation approach of the microorganism characterized by making it freeze or freeze-dry or a cell is offered.
[0007] There are various kinds of things as a microorganism to which this invention is applied, for example, lactic acid bacteria, such as lactobacillus bifidus, such as Bifidobacterium address SENTESU, Bifidobacterium Inn Juan Tess, Bifidobacterium bifidum, Bifidobacterium longum, and Bifidobacterium breve, Streptococcus faecalis, and Lactobacillus acid philus, Escherichia coli like ESSHIERISHIA KORI K-12, a Bacillus subtilis like 168 shares of bacillus Subtilis mull BURUGU, and yeast like Saccharomyces SEREBISHIE can be mentioned.
[0008] Moreover, as an animal cell, the germ of Homo sapiens, a cow, a horse, a goat, the sheep, a rabbit, a hamster, a rat, and a mouse, a cancer cell, a T-cell leukemic cell, a hybridoma, fibrocyte, a blood vessel configuration cell, a bone marrow cell, a distributed islet cell, the cultured cell of fishes, etc. are mentioned. As a plant cell, cultured cells, such as a rice, wheat, a Madagascar periwinkle, corn, a sugarcane, tobacco, lavender, an apple, a ginseng, soybeans, a liverwort, a strawberry, a potato, a carnation, a pea, asparagus, MEKYABETSU, and a pear, a protoplast, a shoot apex, an adventious embryo, etc. can be mentioned.
[0009] After cultivating a microorganism by the culture medium of arbitration, a harvest is carried out according to centrifugal separation etc., the wet fungus body which washed by the suitable penetrant

remover is used, and a cell can use the cell cultivated by the approach of arbitration, such as shaking culture. The obtained microorganism or cell is used as suspension which added the buffer solution remaining as it is or little, and is made to suspend in the diluent containing the inulin mold fructan sterilized beforehand.

[0010] As an active principle, it is independent, or skim milk, dimethyl sulfoxide, a glycerol, grape sugar, cane sugar, a lactose, and vitamins can be combined suitably, and the component of a diluent can add them that it is simultaneous or additionally if needed including an inulin mold fructan as other components, and can be used.

[0011] The concentration of the inulin mold fructan used for this invention is preferably used in 1 - 40% of the weight of the range that what is necessary is just to use it by the concentration suitable for the microorganism to freeze or a cell. Moreover, the inulin mold fructan of this invention can be used as a part or all of a presentation of a frost damage protective agent. a with a polymerization degree of three or more to which the fructose has combined the inulin mold fructan with cane sugar in straight chain fructan -- it is -- usually -- polymerization degree 3-15 -- the thing of polymerization degree 3-6 is independent preferably, or it is used as two or more sorts of mixture.

[0012] Such inulin mold fructan or its mixture can be obtained extracting from chicory, an artichoke, etc., or by making the enzyme which has fructose transition capacity in cane sugar act. Although the following will be mentioned if a concrete inulin mold fructan is illustrated, this invention is not limited to these. For example, the inulin mold fructan obtained by the extract of an artichoke The thing (30 - 50 % of the weight) of polymerization degree 3-6, a with a polymerization degree of seven or more thing (20 - 50 % of the weight), The inulin mold fructan (trade name: MEIORIGO G; Meiji Seika Kaisha, Ltd. make) which have the sugar composition of a monosaccharide and 2 sugar (10 - 30 % of the weight), and the enzyme of the Aspergillus nigre origin is made to act on cane sugar, and is obtained The thing of polymerization degree 3-6 (55 - 60 % of the weight), It has the sugar composition of a monosaccharide and 2 sugar (40 - 45 % of the weight). Furthermore, the fructan mixture which uses the sugar of polymerization degree 3-6 as a principal component can be obtained by carrying out partial purification of the above-mentioned mixture using a column chromatography or the film. For example, by carrying out partial purification of above-mentioned MEIORIGO G using a column chromatography, the inulin mold fructan (trade name: MEIORIGOP) which contains the thing of polymerization degree 3-6 at 95% of the weight or more of a rate can be obtained, and the inulin mold fructan of polymerization degree 3-6 (70 % of the weight or more) can be obtained also from an artichoke extract. Furthermore, the inulin mold fructan 4 (nistose) which uses single polymerization degree as a principal component, for example, polymerization degree, and polymerization degree 5 (cell tosyl nistose) can be acquired by combining a column chromatography, crystallization, etc.

[0013] this invention frost damage protective agent for the microorganism characterized by containing an inulin mold fructan as an active principle or a cell shows the large improvement in a ratio (survival rate) which a microorganism and a cell survive after cryopreservation compared with the known frost damage protective agent containing the active principle known conventionally, and can prevent effectively the extinction in a microorganism or the freezing process of a cell.

[0014]

[Example] Next, although the example of this invention is shown, this is instantiation to the last and this invention is not limited to this.

Anaerobic culture of the 37 degrees C (Bifidobacterium longum) of the example 1 lactobacillus bifidus was carried out by BL culture medium for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- an anaerobic phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation --

the harvest was carried out. The obtained wet fungus body was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) After making the diluent only containing 6.7 % of the weight (inulin mold fructan of polymerization degree 4) of nistose suspend the obtained wet fungus body in homogeneity, it was made to freeze at -25 degrees C, and freezing and fusion (it is rapid fusion at freezing and 30 degrees C in -25 degrees C) were repeated 3 times after 24-hour preservation. Consequently, the numbers of micro organisms of a cryopreservation bacillus were 2.08x108 / ml, and the survival rate was 41.6%.
[0015] (b) After making the diluent only containing 6.7 % of the weight (presentation: 52% of things of polymerization degree 3-10, 34% of with a polymerization degree of 11 or more things) of inulin mold fructans extracted from the artichoke suspend a wet fungus body in homogeneity, the same actuation as said (b) was performed. Consequently, the numbers of micro organisms of a cryopreservation bacillus were 1.25x108 / ml, and the survival rate was 25.5%.
(c) After making the diluent which contains only 6.7 % of the weight (mol concentration 0.1M) of cane sugars as an example of contrast suspend a wet fungus body in homogeneity, when the same actuation as said (b) was performed, the numbers of micro organisms of a cryopreservation bacillus were 6.30x107 / ml, and the survival rate was 12.9%.
[0016] Anaerobic culture of the 37 degrees C (Bifidobacterium address SENTESU) of the example 2 lactobacillus bifidus was carried out by BL culture medium for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- an anaerobic phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained wet fungus body was carried out for equivalent 4 minutes, and the following experiments were presented.
(b) It mixed by the weight ratio 1:1 to the diluent containing 3 % of the weight (inulin mold fructan of polymerization degree 4) of nistose, and 10 % of the weight of skim milk, and the obtained wet fungus body was adjusted to pH7.0 (5 convention sodium-hydroxide solution). Mixed liquor was poured distributively on the petri dish and it freeze-dried at -20 degrees C. The number of micro organisms after freeze drying was 1.18x1011/g, and the survival rate was 33.5%.
[0017] (b) After mixing the diluent and wet fungus body containing the MEIORIGO P(Meiji Seika [Kaisha, Ltd.] make [a trade name,] the presentation: 44.4% [of things of polymerization degree 3], 42.9% [of things of polymerization degree 4], 8.9% [of things of polymerization degree 5], 0.6% of things of polymerization degree 6) 3 % of the weight and 10 % of the weight of skim milk which is the mixture of an inulin mold fructan by the weight ratio 1:1, the same actuation as said (b) was performed. The number of micro organisms after freeze drying was 1.08x1011/g, and the survival rate was 30.6%.
(c) After mixing the diluent and wet fungus body containing 3 % of the weight (inulin mold fructan of polymerization degree 5) of cell tosyl nistose, and 10 % of the weight of skim milk by the weight ratio 1:1, when the same actuation as said (b) was performed, the number of micro organisms after freeze drying was 1.09x1011/g, and the survival rate was 30.8%.
(d) After mixing a wet fungus body by the weight ratio 1:1 to the diluent which contains 3 % of the weight of lactoses, and 10 % of the weight of skim milk as an example of contrast, the same actuation as said (b) was performed. Consequently, the number of micro organisms after freeze drying was 5.88x1010/g, and the survival rate was 16.7%.
[0018] 37 degrees C (ESSHIERISHIA KORI K-12) of example 3 Escherichia coli were cultivated by L broth for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- a phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained

wet fungus body was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) The obtained wet fungus body was suspended at homogeneity in the diluent only containing 10 % of the weight (inulin mold fructan of polymerization degree 4) of nistose, and it froze at -25 degrees C. Freezing and fusion (it is rapid fusion at freezing and 30 degrees C in -25 degrees C) were repeated 3 times after 24-hour preservation. Consequently, the survival rate of the number of micro organisms of a cryopreservation bacillus was 41.6% in 3.99x108 / ml.
[0019] (b) When the diluent only containing 10 % of the weight (inulin mold fructan of polymerization degree 3) of 1-kestose was made to suspend a wet fungus body in homogeneity and the same actuation as said (b) was performed, the numbers of micro organisms of a cryopreservation bacillus were 2.47x108 / ml, and the survival rate was 25.8%.
(c) When the diluent which contains only 10 % of the weight of trehaloses as an example of contrast was made to suspend a wet fungus body in homogeneity and the same actuation as said (b) was performed, the survival rate of the number of micro organisms of a cryopreservation bacillus was 20.2% in 1.86x106 / ml.
(d) The diluent which contains only 10 % of the weight of cane sugars as an example of contrast was made to suspend a wet fungus body in homogeneity, and the same actuation as said (b) was performed. Consequently, the survival rate of the cryopreservation bacillus was 18.3% in the number of micro organisms 1.77x108 / ml.
[0020] 37 degrees C (Lactobacillus acid philus) of example 4 lactic acid bacteria were cultivated by the ILS culture medium for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- a phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained wet fungus body was carried out for equivalent 2 minutes, and the following experiments were presented.
[0021] (b) The wet fungus body was suspended at homogeneity in the diluent only containing 6.7 % of the weight (inulin mold fructan of polymerization degree 4) of nistose, and it froze at -25 degrees C. Freezing and fusion (it is rapid fusion at freezing and 30 degrees C in -25 degrees C) were repeated 3 times after 24-hour preservation. The survival rate of the number of micro organisms of a cryopreservation bacillus was 47.6% in 4.0x108 / ml.
(b) The diluent which contains only 6.7 % of the weight of cane sugars as an example of contrast was made to suspend a wet fungus body in homogeneity, and the same actuation as said (b) was performed. Consequently, the survival rate of the number of micro organisms of a cryopreservation bacillus was 15.7% in 1.32x108 / ml.
[0022] After cultivating at 37 degrees C by the MEM culture medium which added fetal calf serum 10% using the Hela cell of the cancer cell origin as example 5 cultured cell, it processed with trypsin liquid 0.25%, and the cell was removed. This was put into the centrifuging tube, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The obtained cell was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about nistose (inulin mold fructan of polymerization degree 4), it agitated, and the culture medium for freezing was prepared. It enclosed with ampul, after having added and suspended the cell in the culture medium for freezing, putting in the state of coldness and warmth for 1 to 2 hours and making a frost damage protective agent permeate intracellular. This ampul was frozen at -80 degrees C, and was saved for nine days. It diluted with the growth medium about 10 to 20 times after fusion with the 37-degree C thermostat, centrifugal was carried out for 5 - 10 minutes

by 500 - 600rpm, and the cell was collected. The dissolved cell checked life and death using trypan blue dyeing, and as a result of computing a survival rate, it survived 93%.
[0023] (b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glycerol as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 65%.
(c) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glucose as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 62%.
[0024] Ham's which added fetal calf serum 10% using CHO-K1 cell of the Chinese hamster ovary cell origin as example 6 cultured cell After cultivating at 37 degrees C by F12 culture medium, it processed with trypsin liquid 0.25%, and the cell was removed. This was put into the centrifuging tube, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The obtained cell was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about nistose (inulin mold fructan of polymerization degree 4), it agitated, and the culture medium for freezing was prepared. It enclosed with ampul, after having added and suspended the cell in the culture medium for freezing, putting in the state of coldness and warmth for 1 to 2 hours and making a frost damage protective agent permeate intracellular. This ampul was frozen at -80 degrees C, and was saved for nine days. It diluted with the growth medium about 10 to 20 times after fusion with the 37-degree C thermostat, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The dissolved cell survived 88%, as a result of checking life and death using trypan blue dyeing and computing a survival rate.
[0025] (b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glycerol as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 61%.
(c) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glucose as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 48%.
(d) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about dimethyl sulfoxide as an example of contrast, it agitated, and the culture medium for freezing was prepared. The same actuation as said (b) was performed for this. Consequently, the survival rate after freezing / fusion was 78%.
[0026] The callus of the hypocotyl origin of example 7 ginseng was cultivated by the liquid medium of suitable arbitration, it processed in the 0.7M mannitol by meicelase P-1 (a trade name, Meiji Seika Kaisha, Ltd. make), filtration, centrifugal, and washing of were done, and protoplast suspension was obtained. The obtained protoplast was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) You could add to the above-mentioned culture medium, it agitated, filtration and sterilization of were done, and it considered as the culture medium for freezing so that it might become the 20 % of the weight of the last concentration about nistose (inulin mold fructan of a degree of polymerization 4) and might become the 10% of the last concentration about dimethyl sulfoxide. protoplast suspension -- a coldness-and-warmth condition -- this culture medium for freezing -- every [small quantity] -- it

added, agitating quietly. After putting for 30 minutes to about 1 hour and making a frost damage protective agent permeate in a protoplast, it froze at -196 degrees C among liquid nitrogen, and saved for nine days. It diluted with the liquid medium which contains the rapid fusion back with a 37-degree C thermostat, and contains 0.4M mannitol in ice, centrifugal was carried out, and the protoplast was collected. The dissolved protoplast checked life and death using EBANZU blue dyeing, and as a result of computing a survival rate, it survived 52%.

[0027] (b) You could add to the above-mentioned culture medium, it agitated, and filtration and sterilization of were done so that it might become the 20 % of the weight of the last concentration about a glucose and might become the 10% of the last concentration about dimethyl sulfoxide as an example of contrast, and it considered as the culture medium for freezing. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 42%.

(c) You could add to the above-mentioned culture medium, it agitated, and filtration and sterilization of were done so that it might become the 20 % of the weight of the last concentration about shoe cloth and might become the 10% of the last concentration about dimethyl sulfoxide as an example of contrast, and it considered as the culture medium for freezing. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 28%.

[0028]

[Effect of the Invention] If the frost damage protective agent of this invention is used, in case the cell of a microorganism, vegetation, and an animal will be frozen, a freezing failure can be controlled and the survival rate can be raised. Therefore, in fields, such as food stuff industry and a pharmaceutical industry, usefulness of this invention is high.

TECHNICAL FIELD

[Industrial Application] This invention relates to the frost damage protective agent and the cryopreservation approach of making an inulin mold fructan an active principle. Furthermore, the freezing failure encountered in it when freezing the cultured cell of microorganisms, such as lactobacillus bifidus, lactic acid bacteria, and Escherichia coli, or vegetation, and an animal in a detail is controlled, and it is related with the frost damage protective agent and the cryopreservation approach of raising a survival rate.

EFFECT OF THE INVENTION

[Effect of the Invention] If the frost damage protective agent of this invention is used, in case the cell of a microorganism, vegetation, and an animal will be frozen, a freezing failure can be controlled and the survival rate can be raised. Therefore, in fields, such as food stuff industry and a pharmaceutical industry, usefulness of this invention is high.

TECHNICAL PROBLEM

[Description of the Prior Art] Storage and preservation of a microorganism or a cell are not only used for preservation of the network which was only excellent, but the use range is expanding it. For example, by the microorganism, pressure of business of lactobacillus bifidus, the lactic acid bacteria, etc. is carried out as a food material in which the lyophilized cell has the ready intestines effectiveness. Moreover, preservation of the Escherichia coli introduced in the specific gene has been an important technical problem on gene engineering.

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[0004] In order to control the frost damage of a microorganism or a cell, the approach of diluting with the protective agent which can be received physiologically was examined, and it was found out that glycerol is effective (Polge, Nature (Nature), 164 volumes, 666 pages, 1949].). Development of the protective agent for controlling frost damage also after that is performed. For example, in lyophilized-products-izing of lactobacillus bifidus or lactic acid bacteria, the approach using lactulose (JP,52-151787,A), vitamin E (JP,53-5747,B), corn steep liquor (JP,58-104787,A), raw starch (JP,58-149675,A), and a cyclodextrin (JP,63-251080,A) as a protective agent added in order to raise a survival rate is learned. However, using this protective agent in large quantities, the matter of these approaches was expensive, and they had the trouble that palatability was not desirable etc. Moreover, when carrying out cryopreservation of the animal cell, the approach of needing addition of a blood serum in the freezing culture medium, or adding methyl cellulose, trehalose (Patent Publication Heisei No. 501112 [four to] official report) or trehalose, and gelatin (JP,5-7489,A) as a protective agent instead of a blood serum is also established. However, also in these approaches, improvement in a survival rate has been a technical problem.

MEANS

[Means for Solving the Problem] Then, this invention persons completed a header and this invention for the ability of extinction of a microorganism or a cell to be remarkably controlled at the time of cryopreservation or freeze drying by using an inulin mold fructan as a frost damage protective agent in a diluent, as a result of examining many things, in order to control extinction of the microorganism in a freezing process, or a cell.

[0006] After dipping a microorganism or a cell into the solution which contains an inulin mold fructan in the frost damage protective agent list for the microorganism characterized by this invention containing an inulin mold fructan as an active principle, or a cell, the cryopreservation approach of the microorganism characterized by making it freeze or freeze-dry or a cell is offered.

[0007] There are various kinds of things as a microorganism to which this invention is applied, for example, lactic acid bacteria, such as lactobacillus bifidus, such as Bifidobacterium address SENTESU, Bifidobacterium Inn Juan Tess, Bifidobacterium bifidum, Bifidobacterium longum, and Bifidobacterium breve, Streptococcus faecalis, and Lactobacillus acid philus, Escherichia coli like ESSHIERISHIA KORI K-12, a Bacillus subtilis like 168 shares of bacillus Subtilis mull BURUGU,

and yeast like Saccharomyces SEREBISHIE can be mentioned.
[0008] Moreover, as an animal cell, the germ of Homo sapiens, a cow, a horse, a goat, the sheep, a rabbit, a hamster, a rat, and a mouse, a cancer cell, a T-cell leukemic cell, a hybridoma, fibrocyte, a blood vessel configuration cell, a bone marrow cell, a distributed islet cell, the cultured cell of fishes, etc. are mentioned. As a plant cell, cultured cells, such as a rice, wheat, a Madagascar periwinkle, corn, a sugarcane, tobacco, lavender, an apple, a ginseng, soybeans, a liverwort, a strawberry, a potato, a carnation, a pea, asparagus, MEKYABETSU, and a pear, a protoplast, a shoot apex, an adventious embryo, etc. can be mentioned.
[0009] After cultivating a microorganism by the culture medium of arbitration, a harvest is carried out according to centrifugal separation etc., the wet fungus body which washed by the suitable penetrant remover is used, and a cell can use the cell cultivated by the approach of arbitration, such as shaking culture. The obtained microorganism or cell is used as suspension which added the buffer solution remaining as it is or little, and is made to suspend in the diluent containing the inulin mold fructan sterilized beforehand.
[0010] As an active principle, it is independent, or skim milk, dimethyl sulfoxide, a glycerol, grape sugar, cane sugar, a lactose, and vitamins can be combined suitably, and the component of a diluent can add them that it is simultaneous or additionally if needed including an inulin mold fructan as other components, and can be used.
[0011] The concentration of the inulin mold fructan used for this invention is preferably used in 1 - 40% of the weight of the range that what is necessary is just to use it by the concentration suitable for the microorganism to freeze or a cell. Moreover, the inulin mold fructan of this invention can be used as a part or all of a presentation of a frost damage protective agent. a with a polymerization degree of three or more to which the fructose has combined the inulin mold fructan with cane sugar in straight chain fructan -- it is -- usually -- polymerization degree 3-15 -- the thing of polymerization degree 3-6 is independent preferably, or it is used as two or more sorts of mixture.
[0012] Such inulin mold fructan or its mixture can be obtained extracting from chicory, an artichoke, etc., or by making the enzyme which has fructose transition capacity in cane sugar act. Although the following will be mentioned if a concrete inulin mold fructan is illustrated, this invention is not limited to these. For example, the inulin mold fructan obtained by the extract of an artichoke The thing (30 - 50 % of the weight) of polymerization degree 3-6, a with a polymerization degree of seven or more thing (20 - 50 % of the weight), The inulin mold fructan (trade name: MEIORIGO G; Meiji Seika Kaisha, Ltd. make) which have the sugar composition of a monosaccharide and 2 sugar (10 - 30 % of the weight), and the enzyme of the Aspergillus nigre origin is made to act on cane sugar, and is obtained The thing of polymerization degree 3-6 (55 - 60 % of the weight), It has the sugar composition of a monosaccharide and 2 sugar (40 - 45 % of the weight). Furthermore, the fructan mixture which uses the sugar of polymerization degree 3-6 as a principal component can be obtained by carrying out partial purification of the above-mentioned mixture using a column chromatography or the film. For example, by carrying out partial purification of above-mentioned MEIORIGO G using a column chromatography, the inulin mold fructan (trade name: MEIORIGOP) which contains the thing of polymerization degree 3-6 at 95% of the weight or more of a rate can be obtained, and the inulin mold fructan of polymerization degree 3-6 (70 % of the weight or more) can be obtained also from an artichoke extract. Furthermore, the inulin mold fructan 4 (nistose) which uses single polymerization degree as a principal component, for example, polymerization degree, and polymerization degree 5 (cell tosyl nistose) can be acquired by combining a column chromatography, crystallization, etc.
[0013] this invention frost damage protective agent for the microorganism characterized by containing an inulin mold fructan as an active principle or a cell shows the large improvement in a ratio (survival

rate) which a microorganism and a cell survive after cryopreservation compared with the known frost damage protective agent containing the active principle known conventionally, and can prevent effectively the extinction in a microorganism or the freezing process of a cell.

EXAMPLE

[Example] Next, although the example of this invention is shown, this is instantiation to the last and this invention is not limited to this.
Anaerobic culture of the 37 degrees C (Bifidobacterium longum) of the example 1 lactobacillus bifidus was carried out by BL culture medium for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- an anaerobic phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained wet fungus body was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) After making the diluent only containing 6.7 % of the weight (inulin mold fructan of polymerization degree 4) of nistose suspend the obtained wet fungus body in homogeneity, it was made to freeze at -25 degrees C, and freezing and fusion (it is rapid fusion at freezing and 30 degrees C in -25 degrees C) were repeated 3 times after 24-hour preservation. Consequently, the numbers of micro organisms of a cryopreservation bacillus were 2.08x108 / ml, and the survival rate was 41.6%.
[0015] (b) After making the diluent only containing 6.7 % of the weight (presentation: 52% of things of polymerization degree 3-10, 34% of with a polymerization degree of 11 or more things) of inulin mold fructans extracted from the artichoke suspend a wet fungus body in homogeneity, the same actuation as said (b) was performed. Consequently, the numbers of micro organisms of a cryopreservation bacillus were 1.25x108 / ml, and the survival rate was 25.5%.
(c) After making the diluent which contains only 6.7 % of the weight (mol concentration 0.1M) of cane sugars as an example of contrast suspend a wet fungus body in homogeneity, when the same actuation as said (b) was performed, the numbers of micro organisms of a cryopreservation bacillus were 6.30x107 / ml, and the survival rate was 12.9%.
[0016] Anaerobic culture of the 37 degrees C (Bifidobacterium address SENTESU) of the example 2 lactobacillus bifidus was carried out by BL culture medium for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- an anaerobic phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained wet fungus body was carried out for equivalent 4 minutes, and the following experiments were presented.
(b) It mixed by the weight ratio 1:1 to the diluent containing 3 % of the weight (inulin mold fructan of polymerization degree 4) of nistose, and 10 % of the weight of skim milk, and the obtained wet fungus body was adjusted to pH7.0 (5 convention sodium-hydroxide solution). Mixed liquor was poured distributively on the petri dish and it freeze-dried at -20 degrees C. The number of micro organisms after freeze drying was 1.18x1011/g, and the survival rate was 33.5%.
[0017] (b) After mixing the diluent and wet fungus body containing the MEIORIGO P(Meiji Seika [Kaisha, Ltd.] make [a trade name,] the presentation: 44.4% [of things of polymerization degree 3], 42.9% [of things of polymerization degree 4], 8.9% [of things of polymerization degree 5], 0.6% of things of polymerization degree 6) 3 % of the weight and 10 % of the weight of skim milk which is the mixture of an inulin mold fructan by the weight ratio 1:1, the same actuation as said (b) was

performed. The number of micro organisms after freeze drying was 1.08x1011/g, and the survival rate was 30.6%.
(c) After mixing the diluent and wet fungus body containing 3 % of the weight (inulin mold fructan of polymerization degree 5) of cell tosyl nistose, and 10 % of the weight of skim milk by the weight ratio 1:1, when the same actuation as said (b) was performed, the number of micro organisms after freeze drying was 1.09x1011/g, and the survival rate was 30.8%.
(d) After mixing a wet fungus body by the weight ratio 1:1 to the diluent which contains 3 % of the weight of lactoses, and 10 % of the weight of skim milk as an example of contrast, the same actuation as said (b) was performed. Consequently, the number of micro organisms after freeze drying was 5.88x1010/g, and the survival rate was 16.7%.
[0018] 37 degrees C (ESSHIERISHIA KORI K-12) of example 3 Escherichia coli were cultivated by L broth for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- a phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained wet fungus body was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) The obtained wet fungus body was suspended at homogeneity in the diluent only containing 10 % of the weight (inulin mold fructan of polymerization degree 4) of nistose, and it froze at -25 degrees C. Freezing and fusion (it is rapid fusion at freezing and 30 degrees C in -25 degrees C) were repeated 3 times after 24-hour preservation. Consequently, the survival rate of the number of micro organisms of a cryopreservation bacillus was 41.6% in 3.99x108 / ml.
[0019] (b) When the diluent only containing 10 % of the weight (inulin mold fructan of polymerization degree 3) of 1-kestose was made to suspend a wet fungus body in homogeneity and the same actuation as said (b) was performed, the numbers of micro organisms of a cryopreservation bacillus were 2.47x108 / ml, and the survival rate was 25.8%.
(c) When the diluent which contains only 10 % of the weight of trehaloses as an example of contrast was made to suspend a wet fungus body in homogeneity and the same actuation as said (b) was performed, the survival rate of the number of micro organisms of a cryopreservation bacillus was 20.2% in 1.86x106 / ml.
(d) The diluent which contains only 10 % of the weight of cane sugars as an example of contrast was made to suspend a wet fungus body in homogeneity, and the same actuation as said (b) was performed. Consequently, the survival rate of the cryopreservation bacillus was 18.3% in the number of micro organisms 1.77x108 / ml.
[0020] 37 degrees C (Lactobacillus acid philus) of example 4 lactic acid bacteria were cultivated by the ILS culture medium for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- a phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained wet fungus body was carried out for equivalent 2 minutes, and the following experiments were presented.
[0021] (b) The wet fungus body was suspended at homogeneity in the diluent only containing 6.7 % of the weight (inulin mold fructan of polymerization degree 4) of nistose, and it froze at -25 degrees C. Freezing and fusion (it is rapid fusion at freezing and 30 degrees C in -25 degrees C) were repeated 3 times after 24-hour preservation. The survival rate of the number of micro organisms of a cryopreservation bacillus was 47.6% in 4.0x108 / ml.
(b) The diluent which contains only 6.7 % of the weight of cane sugars as an example of contrast was made to suspend a wet fungus body in homogeneity, and the same actuation as said (b) was performed.

Consequently, the survival rate of the number of micro organisms of a cryopreservation bacillus was 15.7% in 1.32x108 / ml.

[0022] After cultivating at 37 degrees C by the MEM culture medium which added fetal calf serum 10% using the Hela cell of the cancer cell origin as example 5 cultured cell, it processed with trypsin liquid 0.25%, and the cell was removed. This was put into the centrifuging tube, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The obtained cell was carried out for equivalent 3 minutes, and the following experiments were presented.

(b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about nistose (inulin mold fructan of polymerization degree 4), it agitated, and the culture medium for freezing was prepared. It enclosed with ampul, after having added and suspended the cell in the culture medium for freezing, putting in the state of coldness and warmth for 1 to 2 hours and making a frost damage protective agent permeate intracellular. This ampul was frozen at -80 degrees C, and was saved for nine days. It diluted with the growth medium about 10 to 20 times after fusion with the 37-degree C thermostat, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The dissolved cell checked life and death using trypan blue dyeing, and as a result of computing a survival rate, it survived 93%.

[0023] (b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glycerol as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 65%.

(c) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glucose as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 62%.

[0024] Ham's which added fetal calf serum 10% using CHO-K1 cell of the Chinese hamster ovary cell origin as example 6 cultured cell After cultivating at 37 degrees C by F12 culture medium, it processed with trypsin liquid 0.25%, and the cell was removed. This was put into the centrifuging tube, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The obtained cell was carried out for equivalent 3 minutes, and the following experiments were presented.

(b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about nistose (inulin mold fructan of polymerization degree 4), it agitated, and the culture medium for freezing was prepared. It enclosed with ampul, after having added and suspended the cell in the culture medium for freezing, putting in the state of coldness and warmth for 1 to 2 hours and making a frost damage protective agent permeate intracellular. This ampul was frozen at -80 degrees C, and was saved for nine days. It diluted with the growth medium about 10 to 20 times after fusion with the 37-degree C thermostat, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The dissolved cell survived 88%, as a result of checking life and death using trypan blue dyeing and computing a survival rate.

[0025] (b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glycerol as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 61%.

(c) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glucose as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 48%.

(d) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about dimethyl sulfoxide as an example of contrast, it agitated, and the culture medium for freezing was prepared. The same actuation as said (b) was performed for this. Consequently, the survival rate after freezing / fusion was 78%.

[0026] The callus of the hypocotyl origin of example 7 ginseng was cultivated by the liquid medium of suitable arbitration, it processed in the 0.7M mannitol by meicelase P-1 (a trade name, Meiji Seika Kaisha, Ltd. make), filtration, centrifugal, and washing of were done, and protoplast suspension was obtained. The obtained protoplast was carried out for equivalent 3 minutes, and the following experiments were presented.

(b) You could add to the above-mentioned culture medium, it agitated, filtration and sterilization of were done, and it considered as the culture medium for freezing so that it might become the 20 % of the weight of the last concentration about nistose (inulin mold fructan of a degree of polymerization 4) and might become the 10% of the last concentration about dimethyl sulfoxide. protoplast suspension -- a coldness-and-warmth condition -- this culture medium for freezing -- every [small quantity] -- it added, agitating quietly. After putting for 30 minutes to about 1 hour and making a frost damage protective agent permeate in a protoplast, it froze at -196 degrees C among liquid nitrogen, and saved for nine days. It diluted with the liquid medium which contains the rapid fusion back with a 37-degree C thermostat, and contains 0.4M mannitol in ice, centrifugal was carried out, and the protoplast was collected. The dissolved protoplast checked life and death using EBANZU blue dyeing, and as a result of computing a survival rate, it survived 52%.

[0027] (b) You could add to the above-mentioned culture medium, it agitated, and filtration and sterilization of were done so that it might become the 20 % of the weight of the last concentration about a glucose and might become the 10% of the last concentration about dimethyl sulfoxide as an example of contrast, and it considered as the culture medium for freezing. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 42%.

(c) You could add to the above-mentioned culture medium, it agitated, and filtration and sterilization of were done so that it might become the 20 % of the weight of the last concentration about shoe cloth and might become the 10% of the last concentration about dimethyl sulfoxide as an example of contrast, and it considered as the culture medium for freezing. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 28%.